Nuclear re-organisation of the Hoxb complex during mouse embryonic development

Nuclear re-organisation of the *Hoxb* complex during mouse embryonic development

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Summary

The spatial and temporal co-linear expression of Hox genes during development is an exquisite example of programmed gene expression. The precise mechanisms underpinning this are not known. Analysis of *Hoxb* chromatin structure and nuclear organisation, during the differentiation of murine ES cells, has lent support to the idea that there is a progressive 'opening' of chromatin structure propagated through Hox clusters from 3′ to 5′, which contributes to the sequential activation of gene expression. Here, we show that similar events occur in vivo in at least two stages of development. The first changes in chromatin structure and nuclear organisation were detected during gastrulation in the *Hoxb1*-expressing posterior primitive streak region: *Hoxb* chromatin was decondensed and the *Hoxb1* locus looped out from its chromosome territory, in contrast to non-expressing *Hoxb9*, which remained within the chromosome territory. At E9.5, when differential Hox expression along the anteroposterior axis is being established, we found concomitant changes in the organisation of *Hoxb*. *Hoxb* organisation differed between regions of the neural tube that had never expressed *Hoxb* [rhombomeres (r) 1 and 2], strongly expressed *Hoxb1* but not *b9* (r4), had downregulated *Hoxb1* (r5), expressed *Hoxb9* but not *Hoxb1* (spinal cord), and expressed both genes (tail bud). We conclude that *Hoxb* chromatin decondensation and nuclear re-organisation is regulated in different parts of the developing embryo, and at different developmental stages. The differential nuclear organisation of *Hoxb* along the anteroposterior axis of the developing neural tube is coherent with co-linear Hox gene expression. In early development nuclear re-organisation is coupled to *Hoxb* expression, but does not anticipate it.

Key words: Chromatin, Embryonic development, Hox genes, Nuclear organisation, Rhombomere, Mouse

Introduction

In mammals, Hox genes are organised in four clusters, each subject to tight transcriptional regulation. The co-linear expression of Hox genes is a remarkable example of spatial and temporal control of gene expression, and is essential for correct embryonic development (Kmita and Duboule, 2003). Genes located at one (3′) end of a Hox cluster are activated earlier, and in more anterior structures of the embryo, than genes lying progressively toward the 5′ end of the cluster. Transgenic experiments in the mouse have revealed both local (e.g. Marshall et al., 1994), and more distant (Spitz et al., 2003) cis-regulatory elements involved in the control of Hox gene expression. In addition, experiments that transpose Hox genes within and between clusters have led to the suggestion that there is a progressive change in chromatin structure propagated through Hox clusters from 3′ to 5′, that prevents posterior genes from being expressed early during embryonic development. The precise nature of the underlying mechanisms are still unknown, but might involve a progressive 'opening' of chromatin, or a progressive relief from a silencing mechanism (Kmita and Duboule, 2003).

We have previously investigated nuclear organisation and chromatin structure changes at the murine *Hoxb* cluster using a retinoic acid (RA) induced ES cell differentiation model (Chambeyron and Bickmore, 2004). We showed that the progressive transcriptional activation of *Hoxb* genes is associated, not only with a visible decondensation of *Hoxb*, but also with the choreographed extrusion of the genes out of their chromosome territory (CT). These observations lend support to a model in which there is progressive change in large-scale chromatin structure, initiating at the 3′ end, which contributes to the sequential activation of gene expression from this Hox cluster (Kmita and Duboule, 2003; Roelen et al., 2002; Bickmore et al., 2005).

Although the ES cell system seems to recapitulate the temporal activation of *Hoxb* genes (Simeone et al., 1990), it remains unclear whether this activation mechanism, and the concomitant chromatin and nuclear re-organisation, reflect the mechanisms that operate in vivo (Duboule and Deschamps, 2004). Co-linear Hox regulation occurs several times during embryonic development. The first wave of *Hoxb* expression is early in gastrulation, initiating in the most posterior (caudal) part of the primitive streak (PS) (Forlani et al., 2003). Later, towards mid-gestation, the establishment of restricted domains of *Hoxb* expression in the neural tube also depends on co-linearity.

Here, we have used fluorescence in situ hybridisation (FISH) on mouse embryo tissue sections in order to determine whether

the chromatin changes seen at *Hoxb* during ES cell differentiation also occur during different stages of embryogenesis. We show that at the onset of gastrulation the transcriptional activation of *Hoxb1* in the posterior PS is accompanied by chromatin decondensation of *Hoxb* and extrusion of *Hoxb1* from its CT. These events are not seen in the non-expressing extra-embryonic (EE) cells of the same embryos. Decondensation and looping out of *Hoxb1* is also seen in cells of rhombomere 4 (r4) at E9.5, but not in rhombomeres 1 or 2, anterior to r4, or in r5. By contrast a more 5′ (non-expressed) gene (*Hoxb9*) does not loop out from the CT in these *Hoxb1*-expressing cells. We do not detect significant chromatin decondensation of *Hoxb* in the neural tube at E9.5 posterior to the hindbrain, but there is movement of *Hoxb9* out from the CT in the spinal cord, where this gene is expressed. By contrast, *Hoxb1* and *Hoxb9* both located at the edge of their CT in the spinal cord of the tailbud region, where they are co-expressed.

Last, we show that chromatin decondensation and movement of *Hoxb1* does not occur prior to transcriptional activation, either in the cells of the posterior streak region (PSR) of E6.5 embryos, which can autonomously express *Hoxb1* in explants (Forlani et al, 2003), or the cells of the posterior epiblast (PEP) that can precociously activate *Hoxb1* expression in response to RA (Roelen et al., 2002).

We conclude that the programmed events of chromatin decondensation and nuclear re-organisation, seen at the *Hoxb* complex during ES cell differentiation ex vivo, are reproduced in vivo in at least two distinct stages of development: primitive streak formation and patterning of the neural tube. Nuclear re-organisation is coupled to *Hoxb* expression, and does not anticipate it during early development. Moreover, there is differential nuclear organisation of *Hoxb* along the anteroposterior axis of the neural tube, which parallels the co-linear expression of Hoxb genes. These data are consistent with nuclear re-organisation being part of a developmental mechanism involved in the co-linear regulation of Hox genes.

Materials and methods

Mouse embryo sectioning and staging

Embryos were collected from crosses between *Dct-Lacz* homozygous transgenic CD1×CD1 mice (MacKenzie et al., 1997). We confirmed, by FISH, that the *Dct-Lacz* transgene is on a different mouse chromosome from *Hoxb* (data not shown). The day on which the vaginal plug was detected was considered 0.5 days of gestation (E0.5).

E6.5 and E7.5 embryos in the decidua, and E9.5 embryos, were fixed with 4% formaldehyde overnight at 4°C, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin blocks. Adjacent serial sections were cut at 4 μm and used for FISH, immunohistochemistry or staining with Haematoxylin and Eosin (HE). HE stained sections from E6.5 and E7.5 embryos were used to stage embryos according to criteria previously published (Downs and Davies, 1993).

Immunohistochemistry

Immunohistochemistry on 4 μm paraffin sections was performed with Vectastain peroxidase staining kit (Vector Laboratories) according to manufacturer's instruction using a 1:200 dilution of a polyclonal antibody against Hoxb1 (Covance). For epitope unmasking, sections were treated by microwave (900 W) for 20 minutes in 100 mM TrisHCl pH 10 and subsequently blocked in 90% FCS blocking solution. Sections were counterstained with 0.75% Eosin for 4 minutes and mounted in Histomount.

Whole-mount in situ hybridisation

Antisense probes for *Hoxb1* and *Hoxb9* were prepared from cDNAs kindly provided by Robb Krumlauf. The *Hoxb1* probe was a T3 transcript from a 0.9 kb *Eco*RI fragment. The *Hoxb9* probe was a T7 polymerase transcript from a 1.3 kb *Eco*RI fragment. The probes were labelled with digoxigenin by in vitro transcription (Roche).

FISH

The protocol for FISH on mouse tissue sections was adapted from (Newsome et al., 2003). Briefly, 4 μm sections were laid on Superfrost slides and were heated to 60°C for 20 minutes, washed four times in xylene for 10 minutes each before dehydration through an ethanol series (100%, 90%, 70%). They were then microwaved for 20 minutes in 0.1 M citrate buffer, pH 6.0. Slides were cooled in buffer for 20 minutes, washed and stored in water. Before use, they were rinsed in 2×SSC, incubated in 2×SSC for 5 minutes at 75°C, denatured for 3 minutes at 75°C in 70% formamide/2×SSC, plunged into ice-cold 70% ethanol for 3 minutes, dehydrated through an alcohol series and air-dried.

Probes were labelled for FISH as described previously (Mahy et al., 2002b; Chambeyron and Bickmore, 2004). Approximately 200 ng of chromosome paint, and 250 ng BAC or plasmid were used per slide with 15 μg mouse C*ot*1 DNA (GibcoBRL) and 5 μg sonicated salmon sperm DNA (sssDNA).

Image capture and analysis

A focus motor was used to collect image stacks of sections on slides, at 0.5 μm intervals along the *z*-axis, using a Zeiss Axioplan fluorescence microscope. Images were captured using a Princeton Instruments Micromax CCD camera, and deconvolved using Hazebuster software (Scanalytics). A three-dimensional (3D) image was reconstructed and analysed using IPLab (Scanalytics).

The distance (d) between two probes in 3D was determined by a more automated approach than previously described (Chambeyron and Bickmore, 2004). Previously, a region containing both probe signals was selected and their segmentation was performed manually. Here, each probe signal is individually selected and a script then determines the segmentation levels. Briefly, a maximum pixel projection was made from the deconvolved image stack and the two probe hybridisation signals were manually delimited. The *xyz* coordinates of the weighted signal centroid were determined for each probe, and d was the distance between the two centroids at opposite corners of a cuboid (Chambeyron and Bickmore, 2004).

Probe localisation relative to the CT in the *z* stack was determined visually. When the probe was not in the same *z* panel as the CT, it was considered outside the CT, and was attributed an arbitrary value of –0.5 μm (the interval between two *z* frames). Where the probe and the CT were in the same *z*-plane, the position of the probe relative to the nearest edge of the CT was determined as previously described for 2D analyses (Mahy et al., 2002a; Chambeyron and Bickmore, 2004).

A two-tailed distribution Student's *t*-test was used to test the statistical significance of differences in chromatin compaction and probe position with respect to CTs. Where there was clearly a non-normal distribution of data, chi-square analysis was used.

Results

Chromatin decondensation of *Hoxb* occurs upon the initiation of transcription in the primitive streak

In our previous ES cell differentiation study we showed that there is a transient decondensation of *Hoxb*, between *Hoxb1* and *Hoxb9*, upon induction of transcription with RA

(Chambeyron and Bickmore, 2004). During mouse embryogenesis proper, *Hoxb1* expression first appears at the late midstreak stage (~E7.0) (Roelen et al., 2002). Expression begins in the nascent mesoderm associated with the most caudal part of the primitive streak (PS), at the boundary between extra-embryonic (EE) and embryonic tissues, and then extends rostrally along, and lateral to, the PS (Forlani et al., 2003). *Hoxb9* expression begins at headfold stage (~E7.5) in the posterior embryonic ectoderm just lateral to the PS (Conlon and Rossant, 1992).

To determine if there is also a decondensation of *Hoxb* chromatin upon the induction of transcription in vivo, we used FISH to determine the interphase separation between *Hoxb1* and *Hoxb9* in tissue sections of E7.5 embryos. According to the published literature, *Hoxb1* expression should be seen from late streak stage onwards, whereas *Hoxb9* expression should not appear until late neural plate/early head fold stage (Conlon and Rossant, 1992). To ensure that only embryos expressing *Hoxb1*, and not *Hoxb9*, were analysed, we selected late streak early bud and neural plate stage embryos. We confirmed the patterns of *Hoxb1* and *Hoxb9* expression by RNA in situ hybridisation (Fig. 1A-C) (Table 1). Hoxb1 protein was also analysed in sagittal sections of E7.5 embryos using a Hoxb1 antibody (Fig. 1F). This confirmed the expression of Hoxb1 in the cells of the primitive streak and the adjacent mesoderm (PSM) (Forlani et al., 2003).

Hoxb1 and *Hoxb9* are separated by 90 kb of DNA (Chambeyron and Bickmore, 2004). We measured the distance d (in μm) between *Hoxb*1 and *Hoxb9* FISH signals in nuclei from E7.5 embryo tissue sections adjacent to those used for expression analysis (Fig. 1G). Chromatin condensation was assessed by a comparison of d^2 values (van den Engh et al., 1992; Chambeyron and Bickmore, 2004). We first examined nuclei from anterior EE yolk sac mesoderm cells (EEM), that are located far from the allantois, and that do not express *Hoxb1*. *Hoxb*1 and *Hoxb9* hybridisation signals are barely separable ($<d^2>=0.08\pm0.02$ μm^2) (Fig. 2A). However, in nuclei from the PSM at E7.5, where *Hoxb1* is expressed (Fig. 1A,F), *Hoxb1* and *Hoxb9* signals are visually separable (Fig. 2A), and the $<d^2>$ (0.2 ± 0.02 μm^2) is significantly larger than that seen in the EEM ($P<0.000$) (Fig. 2B).

These data suggest that, as during ES cell differentiation, there is a visible decondensation of *Hoxb* chromatin in the cells of the embryo as they first express *Hoxb1* at gastrulation. Indeed, we re-measured *Hoxb1-Hoxb9* interphase distances in ES cells after 2 days of differentiation with RA (Chambeyron and Bickmore, 2004), with the script used for analysis of embryo sections described here. The resulting $<d^2>$ (0.25 ± 0.03 μm^2) (Fig. 2B) is remarkably similar to that measured in cells from the E7.5 PSM.

Movement outside of the chromosome territory accompanies *Hoxb1* expression in the primitive streak region

Induction of *Hoxb* expression in ES cells is accompanied, not only by a decondensation of the locus, but also by a choreographed looping out from the CT of the expressed genes (Chambeyron and Bickmore, 2004). To determine if this nuclear re-organisation also occurs during *Hoxb* expression in early embryogenesis, we performed FISH on E7.5 embryo tissue sections, adjacent to those used for

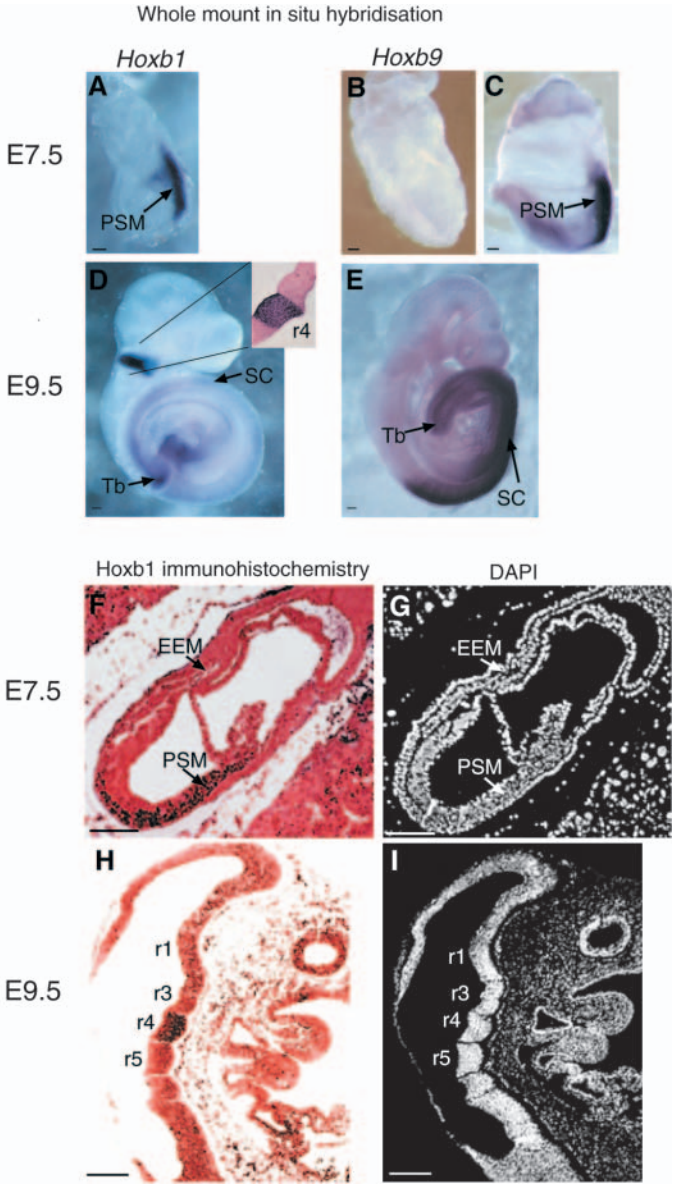

Fig. 1. Expression of *Hoxb1* and *Hoxb9* during mouse embryogenesis. (A-C) Whole-mount in situ hybridisation to detect *Hoxb1* (A) or *Hoxb9* (B,C) expression in E7.5 embryos. *Hoxb1* expression in the primitive streak and adjacent mesoderm (PSM) is seen in a late streak/early bud embryo (A). No *Hoxb9* expression is detected in late neural plate stage embryos (B), but is detected in PSM of headfold stage E7.5 embryos (C). (D,E) Whole-mount in situ hybridisation to detect *Hoxb1* (D) or *Hoxb9* (E) expression in E9.5 embryos. *Hoxb1* expression is seen in the tailbud (Tb) and in a segment of the hindbrain (D). A sagittal section of this embryo shows that this latter region is rhombomere 4 (r4) (shown in inset at higher magnification). There is no *Hoxb1* expression in the anterior region of the spinal cord (SC). *Hoxb9* expression (E) is seen in the SC and Tb, but not in the hindbrain. (F) Immunohistochemistry staining of a near sagittal section from an E7.5 (neural plate stage) embryo, with antibody that recognises Hoxb1, and counterstained with Eosin. Hoxb1 is seen in the PSM. There is no staining in extra-embryonic mesoderm (EEM). (G) DAPI staining of an adjacent E7.5 section that was used for FISH analysis. (H) Immunohistochemistry staining for Hoxb1 on a sagittal section from an E9.5 embryo. The positions of rhombomeres 1 to 4 (r1-4) are indicated. (I) DAPI staining of an adjacent E9.5 section that was used for FISH analysis. Scale bars: 200 μm.

Table 1. Expression patterns of *Hoxb1* and *Hoxb9* during embryogenesis

Embryonic stage	Tissue	Expression	
		Hoxb1	*Hoxb9*
E6.5	Distal region	–	–
	Posterior streak region	–	–
		(autonomous expression in explants)	
	Anterior epiblast	–	–
	Posterior epiblast	–	
		(but responsive to RA)	–
E7.5 (late streak and early bud stage)	Extra-embryonic; anterior yolk sac mesoderm; extra-embryonic ectoderm	–	–
	Primitive streak + adjacent embryonic mesoderm	+	–
E9.5	Rhombomere 1 or 2	–	–
	Rhombomere 4	+	–
	Rhombomere 5	Repressed	–
	Spinal cord	Repressed	+
	Tailbud	+	+

Expression patterns of *Hoxb1* and *Hoxb9* in different tissues of the mouse embryo from E6.5 to E9.5. –, not expressed; +, expressed; repressed, no longer expressed.

immunohistochemistry, using *Hoxb1* or *Hoxb9* probes, and a paint for mouse chromosome 11 (MMU11) (Fig. 3A,B). We measured the distance (μm) between the *Hoxb* gene signals and the nearest CT edge in nuclei from extra-embryonic cells and PSM (Mahy et al., 2002a; Chambeyron and Bickmore, 2004).

In both the EE mesoderm (EEM), that comes from the primitive streak, and the EE ectoderm (EEE), that is not from a region with the potential to express *Hoxb1*, most (70%) *Hoxb1* hybridisation signals were at the edge of, or inside of, the CT (Fig. 3C). However in the PSM, 47% of *Hoxb1* hybridisation signals were outside of the CT (Fig. 3C). A comparison of the mean positions of *Hoxb1* in EEM and EEE, with that in the PSM, shows that there is a significant (P=0.025) movement of *Hoxb1* outside of its CT in the expressing PSM tissue (Fig. 3D). By contrast, *Hoxb9* is not expressed anywhere in pre-late neural plate stage E7.5 embryos, and most (66 and 73%) *Hoxb9* alleles remain inside of the CT in both the EE and PSM (P=0.8) (Fig. 3C,D).

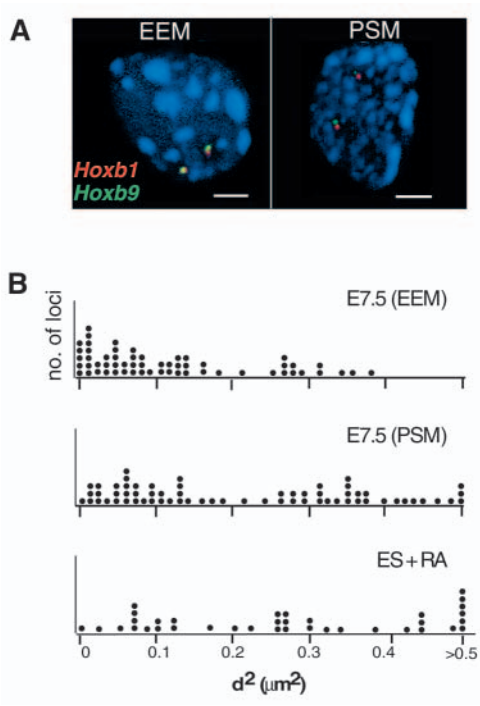

Fig. 2. Chromatin decondensation at *Hoxb* during early embryogenesis. (A) FISH of *Hoxb1* (red) and *Hoxb9* (green) on nuclei from extra embryonic mesoderm (EEM) and primitive streak/adjacent mesoderm (PSM) of one E7.5 embryo. Nuclei were counterstained with DAPI (blue). Scale bar: 2 μm. (B) Distribution of squared interphase distances (d^2) in μm^2 measured between probes for *Hoxb1* and *Hoxb9*, on nuclei from EEM and PSM of one E7.5 embryo, and in ES cells differentiated with RA for 2 days.

Therefore, as in ES cells, there is a specific extrusion of the 3′ end (*Hoxb1*) of the *Hoxb* cluster out of the MMU11 CT when *Hoxb1* expression initiates in embryogenesis. Another similarity that we observed with the ES system is that, even in non-expressing tissues (EEM and EEE), *Hoxb1* is located closer to the CT surface than *Hoxb9* is (Fig. 3D).

Chromatin decondensation and nuclear organisation of *Hoxb* during hindbrain segmentation

Later in embryogenesis, *Hoxb* genes show spatially restricted patterns of gene expression within segments of the developing hindbrain. Expression of *Hoxb1* mRNA (Murphy et al., 1989; Wilkinson et al., 1989) and protein (Ferretti et al., 1999; Arenkiel et al., 2004) is restricted to rhombomere 4 (r4) of the hindbrain at E9.5 (Fig. 1D,H). However, these cells are not descendants of the expressing cells from the E7.5 PS (Forlani et al., 2003). Rather, *Hoxb1* expression in r4 is the result of a separate induction of expression. We therefore determined whether *Hoxb1* expression in r4 is accompanied by chromatin and nuclear re-organisation events similar to those seen in ES cells and in the PSM.

The interphase separation of *Hoxb1* and *Hoxb9* in rhombomeres 1 or 2 (r1/r2), where neither gene is expressed, is very similar to that in EEM of E7.5 embryos (Fig. 4A). There was decondensation of *Hoxb* chromatin in r4, but interestingly the distribution of d^2 values in these cells did not follow a normal distribution. Instead, 21% of the loci have a highly decondensed chromatin fibre ($<d^2>=1$ μm^2) whereas 80% remain more condensed ($<d^2>$ 0.02 μm^2) (Fig. 4A). This suggests that not all cells of r4 were transcribing *Hoxb1* at the time of analysis. A chi-squared test showed that the difference

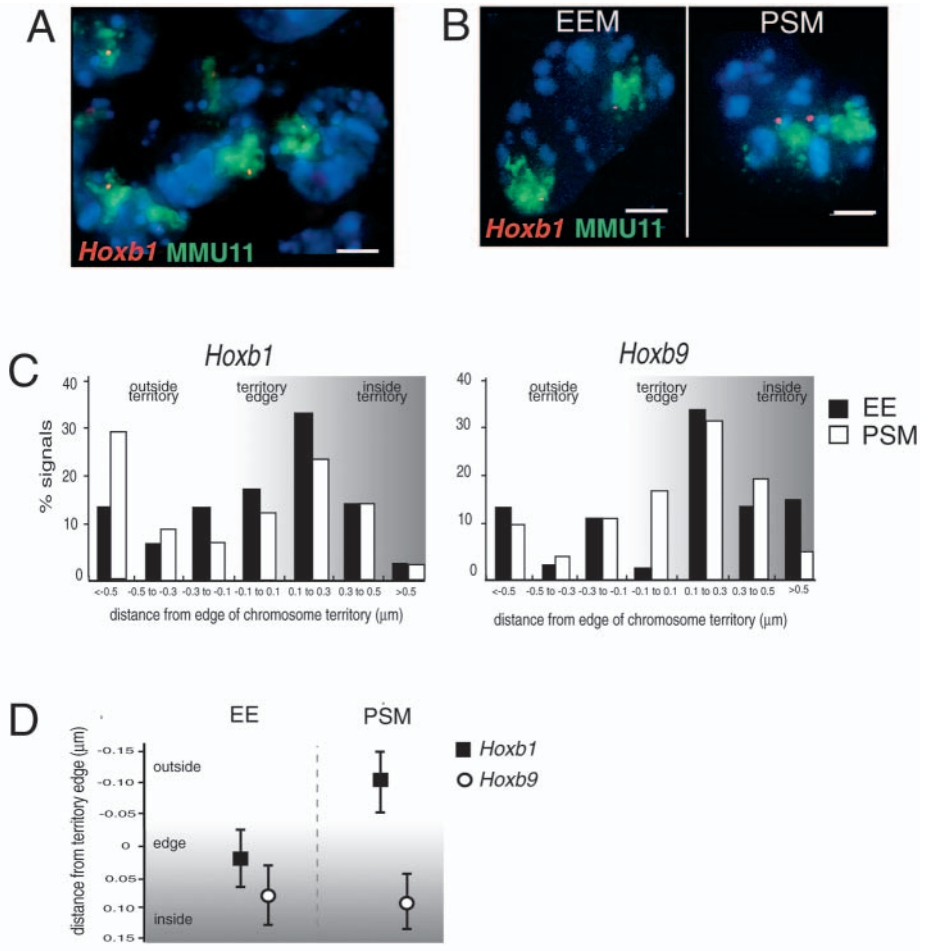

Fig. 3. Nuclear re-organisation of *Hoxb1* during early embryogenesis. (A) FISH with MMU11 chromosome paint (green) and *Hoxb1* probe (red) on a field of nuclei from E7.5 primitive streak regions. The image is a maximal pixel intensity projection from a 3D image stack. (B) Single *z*-plane images from FISH as in A, of single nuclei from E7.5 EEM and PSM. Nuclei were counterstained with DAPI (blue). Scale bars: 2 µm. (C) Histograms showing the position of *Hoxb1* and *Hoxb9* hybridisation signals, relative to the inside, edge or outside of the MMU11 territory, in nuclei from E7.5 EE cells (black bars) and PSM (white bars). The EE and PSM analysed were from two and three embryos from different litters in the case of *Hoxb1*, and from one embryo for *Hoxb9*. Negative distances indicate signals localised beyond the visible limits of the detectable CT. For *Hoxb1*, data from EEM and EEE have been pooled together ($n \geq 60$). (D) Position (mean±s.e.m.) of *Hoxb1* (black squares) and *Hoxb9* (white circles) relative to the inside, edge or outside of the MMU11 territory in nuclei from EE and PSM at E7.5 ($n \geq 60$).

in the distribution of d^2 values between r1/r2 and r4 is statistically significant ($P<0000$). Chromatin decondensation in r4 is also accompanied by a significant ($P=0.015$) re-localisation of *Hoxb1* outside its CT, compared with r1/r2 (Fig. 4B).

There is no expression of *Hoxb1* in r5, immediately posterior to r4, at E9.5 (Fig. 1C). *Hoxb* chromatin is recondensed in this rhombomere, and in particular the population of highly decondensed loci that were seen in r4, is not present (Fig. 4A). *Hoxb1* also re-localises back towards the surface of the CT in r5 cells (Fig. 4B).

Therefore, even though separate events lead to the induction of expression of *Hoxb1* in the PS early in development (E7.5), and in r4 later on (E9.5), both appear to be accompanied by chromatin decondensation and an extrusion of *Hoxb1* out of the CT. By contrast, *Hoxb9* is not expressed anywhere in the hindbrain, and we found that in both r4 and r5, most (66%) *Hoxb9* hybridisation signals are located within the CT (Fig. 4C).

Nuclear organisation of *Hoxb* in the spinal cord

During RA-induced ES cell differentiation, we were able to activate the more 5′ *Hoxb9* gene, but at a later time (day 10) than *Hoxb1*. At that time point we saw movement of *Hoxb9* out of the interior of the CT to a position at the CT surface, or just beyond it. However, there was no accompanying visible decondensation of *Hoxb* chromatin (Chambeyron and Bickmore, 2004). In the E9.5 embryo, *Hoxb9* is strongly expressed along the neural tube, posterior to the level of somites 7-8 (Chen and Capecchi, 1997) (Fig. 1E), but *Hoxb1* is not expressed there (Fig. 1D). By contrast, *Hoxb1* (Gofflot et al., 1997) and *Hoxb9* are both expressed in the tailbud region at E9.5 (Fig. 1D,E).

Similar to the situation in *Hoxb9*-expressing differentiated ES cells, we do not detect gross decondensation of *Hoxb* chromatin in the spinal cord (SC); the $\langle d^2 \rangle$ (0.11 µm^2) is not significantly different from that in r5 ($P=0.5$), which is anterior to the limit of *Hoxb9* expression. However, the $\langle d^2 \rangle$ measured in the spinal cord of the tail bud region (Tb) (0.15 µm^2), was also not significantly larger than that in r5 ($P=0.18$) (Fig. 5A). This is the first situation in either ES cells or in the embryo, where *Hoxb1* expression has not been accompanied by cytological levels of chromatin decondensation.

One explanation for this might be that extreme levels of chromatin decondensation are a consequence of the movement of *Hoxb1* far out of the CT, while *Hoxb9* is still resident within it. In the SC we found that, compared with r5, most *Hoxb9* loci had moved away from the CT interior and towards, or just beyond ($P=0.01$), its edge (Fig. 5B). *Hoxb1* remains within the CT in SC cells (Fig. 5C). As in ES cells, *Hoxb9* movement out from the CT is not as large as that of *Hoxb1* in expressing cells such as r4 (Fig. 4). In the Tb we found that, on average, both *Hoxb1* and *Hoxb9* are located just beyond the edge of the CT (Fig. 5D), but the distribution of positions for both these genes is bimodal (Fig. 5B,C).

Chromatin organisation in cells capable of autonomous or precocious activation of Hoxb1

More than 12 hours before overt *Hoxb1* expression, cells from the posterior streak region (PSR) of early streak stage embryos

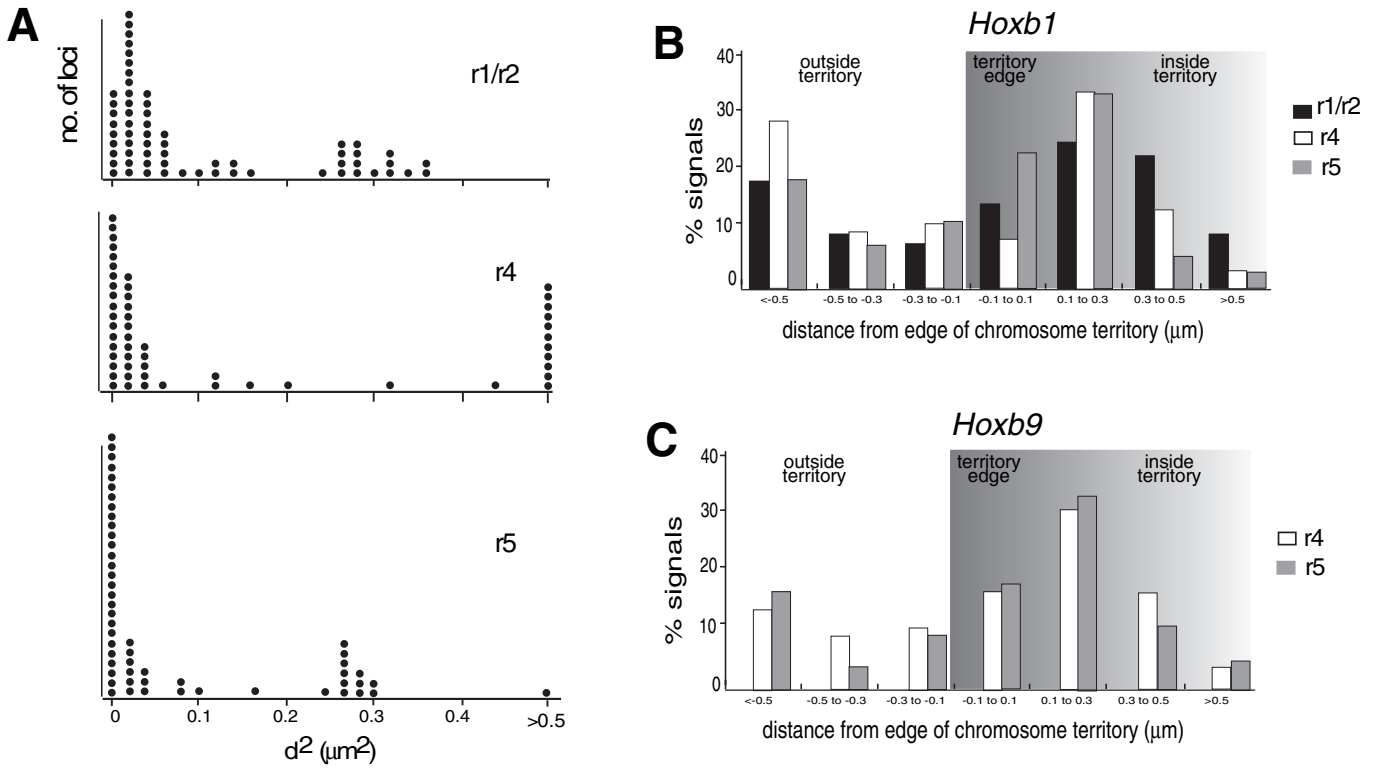

Fig. 4. Chromatin decondensation and nuclear re-organisation of *Hoxb1* in rhombomere 4. (A) The distribution of squared interphase distances (d^2) in μm^2 measured between probes for *Hoxb1* and *Hoxb9*, in rhombomeres 1 and 2 (r1/r2), r4 and r5 from E9.5 embryos. (B) Histogram showing the position (μm) of *Hoxb1* hybridisation signals, relative to the inside, edge or outside of the MMU11 territory, in nuclei from r1/r2 (black bars), r4 (white bars) and r5 (grey bars) of two E9.5 embryos from different litters. Negative distances indicate signals localised beyond the visible limits of the detectable CT ($n \geq 60$). (C) Histogram showing the position of *Hoxb9* hybridisation signals, relative to the MMU11 territory edge, in nuclei from r4 (white bars) and r5 (grey bars) of two E9.5 embryos from different litters. Negative distances indicate signals localised beyond the visible limits of the detectable CT ($n \geq 60$).

(E6.5) can initiate *Hoxb1* expression autonomously in explants (Forlani et al., 2003). By contrast, cells from the distal region (DR) alone cannot. This might suggest some prior opening of *Hoxb* chromatin structure in PSR cells at the onset of gastrulation, which anticipates *Hoxb* expression itself. Therefore, we analysed chromatin condensation and nuclear organisation of *Hoxb* in cells from the PSR (epiblast and mesoderm) and the DR (epiblast) in early streak stage E6.5 embryos (Fig. 6A). Examination of *Hoxb1-Hoxb9* d^2 values indicated that the locus remains condensed in both groups of cells ($<d^2>=0.09\pm0.02$ and 0.10 ± 0.01 μm^2, respectively), similar to EEM cells at E7.5 ($<d^2>=0.08\pm0.02$ μm^2) (Fig. 6B). Similarly, *Hoxb1* remains within its CT in nuclei from both regions at E6.5 (Fig. 6C). However, as in the E7.5 EE, at E6.5 *Hoxb1* is located closer to the surface of the CT than *Hoxb9* (Fig. 6D).

Roelen et al. (Roelen et al., 2002) have shown that, in the E6.5 embryo, a short pulse of exogenous RA can stimulate the precocious expression of *Hoxb1* in the presumptive posterior epiblast (PEP) at the junction between embryonic and EE ectoderm (Fig. 6A). However, we did not find any decondensation of *Hoxb* chromatin in these cells of the embryo either (Fig. 6B). *Hoxb1* was also localised within the CT of these cells (data not shown). We conclude that there is no nuclear re-organisation of *Hoxb* in cells that are destined to express, or that are capable of expressing, *Hoxb1* in the very early embryo. Rather, chromatin opening is tightly coupled to the actual activation of *Hoxb* transcription during gastrulation.

Discussion

Chromatin changes at *Hoxb* during ES cell differentiation recapitulate those that occur early in embryogenesis

We have shown that induction of *Hoxb1* expression in the posterior primitive streak and adjacent mesoderm (E7.5), is accompanied by chromatin decondensation of *Hoxb* (Fig. 2), and by an extrusion of the 3′ end of the cluster (*Hoxb1*) from the chromosome territory (Fig. 3). No remodelling of *Hoxb9* chromatin is seen in cells from pre-late neural plate stages (Fig. 3). This mirrors the polarised *Hoxb* chromatin organisation after 2-4 days of RA-induced ES cell differentiation, when *Hoxb1*, but not *Hoxb9*, is expressed (Chambeyron and Bickmore, 2004). No such re-organisation of *Hoxb* is seen in cells of the EE yolk sac mesoderm, where *Hoxb1* is not expressed. Nor do we detect any evidence of decondensation, or *Hoxb1* movement, in cells of the E6.5 posterior streak region (Fig. 6), a region that does not yet express *Hoxb1*, but that can initiate *Hoxb1* expression autonomously in cultured explants (Forlani et al., 2003). Therefore we conclude that, at the onset of gastrulation, there is no prior chromatin re-modelling at

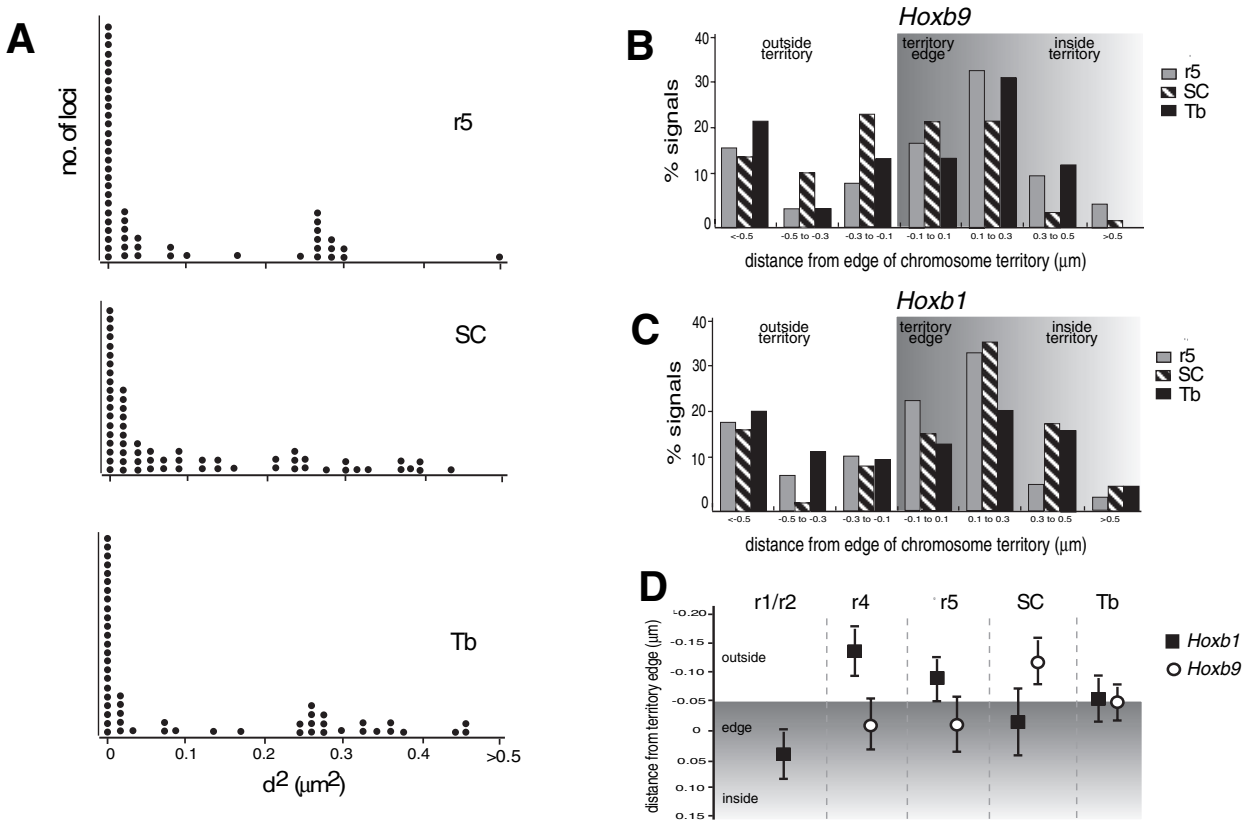

Fig. 5. Nuclear re-organisation of *Hoxb* in the spinal cord. (A) The distribution of squared interphase distances (d^2) in μm^2 measured between probes for *Hoxb1* and *Hoxb9*, in rhombomeres 5 (r5), spinal cord (SC), and tailbud (Tb) from E9.5 embryos. (B,C) Histogram showing the position of *Hoxb9* (B) or *Hoxb1* (C) hybridisation signals, relative to the inside, edge or outside of the MMU11 territory, in nuclei from r5 (grey bars), SC (hatched bars) and Tb (black bars) of two E9.5 embryos from different litters. Negative distances indicate signals localised beyond the visible limits of the detectable CT ($n \geqslant 60$). (D) Positions (mean±s.e.m.) of *Hoxb1* (black squares) and *Hoxb9* (white circles) relative to the inside, edge or outside of the MMU11 territory in nuclei located along the anteroposterior axis of the neural tube at E9.5, from r1/r2 down to the tailbud (Tb) ($n \geqslant 60$).

Hoxb in cells from the region that is destined to later express *Hoxb1*.

However, as in undifferentiated ES cells, *Hoxb1* occupies a position closer to the surface of the CT than *Hoxb9* in early streak stage embryos (Fig. 6). This aspect of *Hoxb* nuclear organisation may have a role in the polarised response of the 3′ end of the cluster to activation in early embryogenesis

Differences in chromatin structure at *Hoxb* in the developing hindbrain

It is perhaps not surprising that ES cells, which are derived from the inner cell mass of the blastocyst, recapitulate the events of *Hoxb1* expression that occur at the primitive streak during gastrulation. However, most of the interest in *Hoxb* expression has been in the anteroposterior spatially restricted segmental expression patterns in the neural tube, which occur later in development. *Hoxb1* is expressed in r4 of the hindbrain of the E9.5 embryo. However, this cannot be the simple result of lineage transmission from the PS cells that first initiate *Hoxb1* expression, because the r4 precursors are already anterior to the node when *Hoxb1* expression spreads caudally from the PS towards the node (Forlani et al., 2003).

Nevertheless we do find some aspects of *Hoxb* nuclear re-organisation replayed in r4 cells. There is movement of *Hoxb1*, but not *Hoxb9*, out from the CT in nuclei from r4. This did not occur in more anterior (r1/r2) segments of the hindbrain (Figs 4 and 5). There is also a decondensation of chromatin at *Hoxb* that is specific to r4, but qualitatively different from that seen in PSM at E7.5. In the latter case, there is a generalised shift in the *Hoxb1-Hoxb9* d^2 to larger values (Fig. 2B), indicating a relatively uniform response of the cell population. This is similar to the decondensation seen in RA-induced ES cells (Fig. 2B). However, the distribution of d^2 values in r4 cells is bimodal (Fig. 4A). Twenty percent of loci are hyper-decondensed ($<d^2>=1.1\pm0.01\ \mu m^2$), but the other alleles are as, or more, condensed as those in r1/r2 ($<d^2>=0.02\pm0.02\ \mu m^2$ and $0.07\pm0.02\ \mu m^2$, respectively). We think this means that only a proportion of cells in r4 will be expressing *Hoxb1* at a given time, perhaps owing to stochastic fluctuation in transcription (Levsky and Singer, 2003; Osborne et al., 2004), or due to changing levels of *Hoxb1* expression in differentiating subpopulations, e.g. motoneurons (Arenkiel et al., 2004). Further investigation of this will necessitate developing assays (e.g. RNA FISH) that allow both transcription and chromatin structure to be analysed in the same nucleus of the embryo.

These data suggest that the precise chromatin changes that occur at *Hoxb1* differ between the early phase of activation at E7.5, and the later segment-restricted phase of expression in

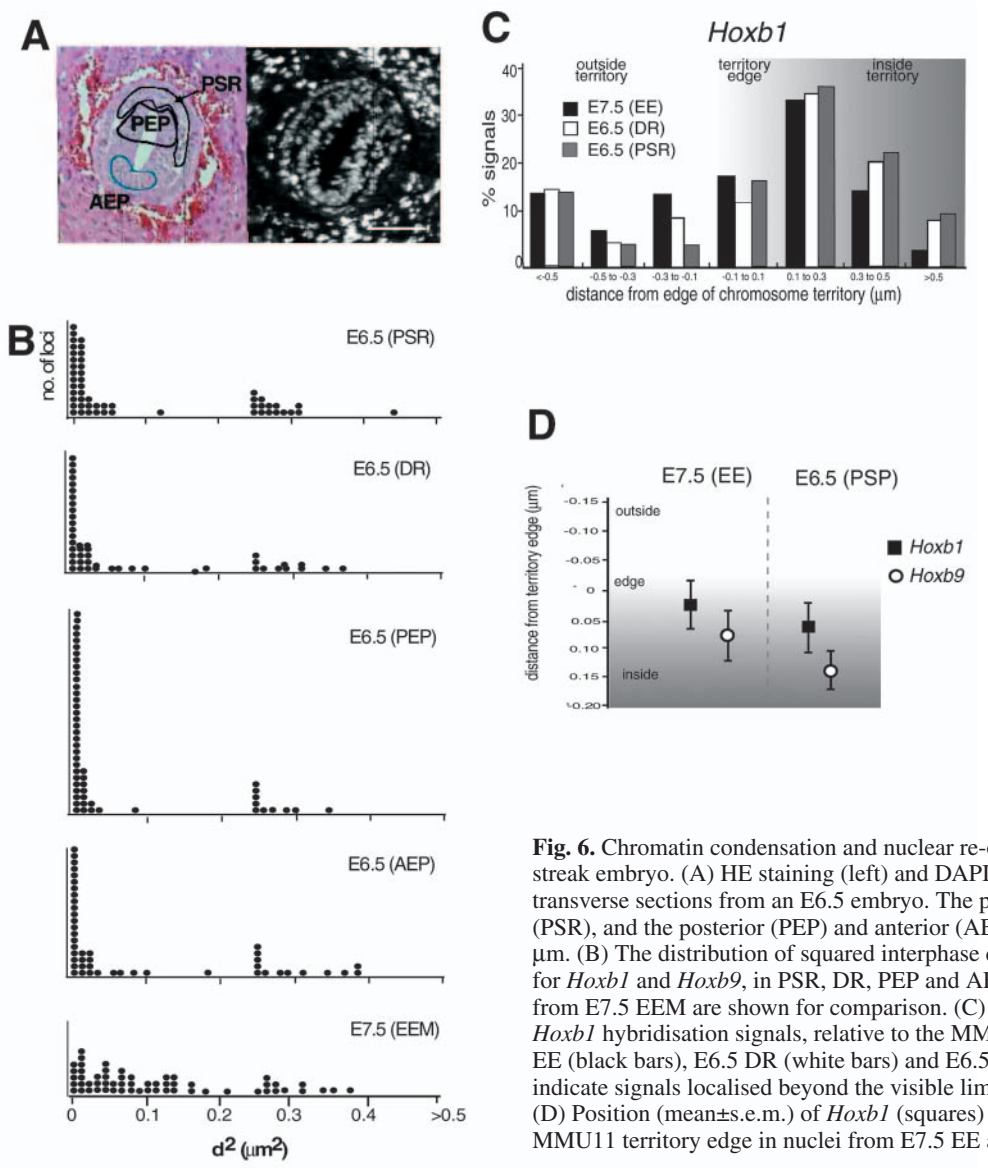

Fig. 6. Chromatin condensation and nuclear re-organisation of *Hoxb* in the E6.5 early streak embryo. (A) HE staining (left) and DAPI counterstaining (right) of serial transverse sections from an E6.5 embryo. The position of the posterior streak region (PSR), and the posterior (PEP) and anterior (AEP) epiblast are indicated. Scale bar: 200 µm. (B) The distribution of squared interphase distances (d^2) measured between probes for *Hoxb1* and *Hoxb9*, in PSR, DR, PEP and AEP of E6.5 embryos. Measurements from E7.5 EEM are shown for comparison. (C) Histogram showing the position of *Hoxb1* hybridisation signals, relative to the MMU11 territory edge, in nuclei from E7.5 EE (black bars), E6.5 DR (white bars) and E6.5 PSR (grey bars). Negative distances indicate signals localised beyond the visible limits of the detectable CT ($n \geq 60$). (D) Position (mean±s.e.m.) of *Hoxb1* (squares) and *Hoxb9* (circles) relative to the MMU11 territory edge in nuclei from E7.5 EE and E6.5 PSR ($n \geq 60$).

the hindbrain. This would be consistent with the behaviour of a *Hoxb1* transgene when transposed to a 5′ position within *Hoxd*. In this case, there was no expression of the transposed transgene in r4, but there was still early mesodermal expression (Kmita et al., 2000). It will now be interesting to analyse chromatin condensation and nuclear organisation of the transposed *Hoxb1* during embryogenesis.

Repression of *Hoxb1* outside of r4

The maintenance of *Hoxb1* expression in r4, and its repression outside r4, depends in part on auto- and cross-regulatory Hox-mediated mechanisms (Popperl et al., 1995; Gavalas et al., 1998; Studer et al., 1998). Specific cis-acting elements and other trans-acting regulators also serve to repress *Hoxb1* transcription in rhombomeres 3 and 5 (Fox, 2000; Giudicelli et al., 2003). It is interesting that although in r5 *Hoxb1* is closer to the edge, or just outside, of the CT, compared with its position in r1/2 cells (Fig. 5D) – perhaps reflecting the history of past *Hoxb1* expression in cells destined to be part of r5 – *Hoxb* chromatin may be more tightly condensed in r5, than in r1/r2 (Fig. 4A) (P=0.09). In addition, the chromatin configuration in r5, may relate to the expression in this hindbrain segment of *Hoxb2*, located only 12 kb 5′ of *Hoxb1* (Wilkinson et al., 1989).

Nuclear organisation of *Hoxb* along the anteroposterior axis of the neural tube.

We did not find any nuclear re-organisation of *Hoxb9* in *b1*-expressing cells at E7.5, and we were careful to restrict our analysis to pre-late neural plate stage embryos that do not express *Hoxb9* (Fig. 1B). However, we do see nuclear re-organisation of *Hoxb9* later in development, at E9.5, in the part of the spinal cord that expresses this gene (Fig. 1E). Compared with r5, in cells from the SC there is a re-localisation of *Hoxb9* loci away from the interior of the CT, to a position at, or just outside, its edge (Fig. 5). This is reminiscent of the situation in ES cells differentiated for 10 days with RA (Chambeyron and Bickmore, 2004).

The tailbud contains descendants of the node and anterior primitive streak (Cambray and Wilson, 2002), and examination

of *Hoxb* nuclear organisation in the spinal cord of this region at E9.5 allowed us to analyse a situation not seen in our ES differentiation system – *Hoxb1* and *b9* co-expression. We do not see *Hoxb* chromatin decondensation in these cells (Fig. 5A) and both *Hoxb1* and *b9* have a similar mean position at the edge of the CT (Fig. 5D). Therefore, there seems to be a different relationship between *Hoxb* expression and nuclear organisation in this part of the neural tube, compared with more anterior regions. In this regard it is interesting to note that the expression of *Hoxb13*, the most 5′ member of the *Hoxb* cluster, and located ~100 kb from *b9*, is restricted to the tailbud region (Zeltser et al., 1996).

We conclude that there is differential nuclear organisation of *Hoxb* along the anteroposterior axis of the neural tube, in a pattern that parallels the spatial pattern of gene expression. To our knowledge, this analysis represents one of the first studies of nuclear organisation in situ within solid tissues of the developing mouse embryo. Combined with the use of mutant embryos it may now provide the opportunity to dissect the mechanisms that bring about chromatin decondensation and nuclear gene movement during mammalian development.

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